Activation of Raf—Mitogen-Activated Protein Kinase Signaling Pathway by 1,25-Dihydroxyvitamin D_3 in Normal Human Keratinocytes

Robert Gniadecki
Department of Dermatological Research, Leo Pharmaceutical Products, Ballerup, Denmark

The biologic effects of the vitamin D hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are believed to be mediated by an intracellular vitamin D receptor, which after ligand binding acts as a transcription factor modulating expression of a variety of genes. Besides having a well-known role in calcium metabolism, this hormone is an important regulator of proliferation in a majority of normal and neoplastic cells. Keratinocytes provide a convenient model for investigating the growth-related effects of vitamin D in normal cells. Growth of keratinocytes may be either stimulated or inhibited by $1,25(OH)_2D_3$, depending on the degree of cell differentiation. We show here that $1,25(OH)_2D_3$ stimulates DNA synthesis via sequential activation of Raf and the mitogen-activated protein kinase. Activation of these kinases is independent on protein and mRNA synthesis and is preceded by rapid tyrosine phosphorylation of an adaptor protein $p66^{Shc}$ and formation of a complex between $p66^{Shc}$, a bridging molecule Grb2, and a Ras activator, mSos. Vitamin D receptor protein associates with Shc, indicating that this steroid hormone is able to signal through the transcription-independent pathways similar to those used by peptide hormones and cytokines. ***Key words: cell cycle/steroid hormones/vitamin D receptor/signal transduction/tyrosine kinase.***
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Research has shown that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) plays an important role in the regulation of cell growth and differentiation (Walters, 1992). In most cell-culture systems, including keratinocytes, $1,25(OH)_2D_3$ blocks proliferation and stimulates cellular differentiation. These biologic responses have been primarily attributed to the activation of the nuclear vitamin D receptor (VDR), which belongs to a superfamily of nuclear steroid receptors together with retinoid A and X receptors and receptors for triiodothyronine. After binding of the ligand, these receptors form homo- or heterodimers with other members of the superfamily and act as transcription factors modulating the expression of a variety of genes (Carlberg *et al*, 1993; Schrader *et al*, 1994). This is referred to as the "genomic" response because this pathway is dependent on gene transcription and new protein synthesis.

Recent evidence suggests that steroid hormones, including $1,25(OH)_2D_3$, may signal independently of the genomic pathway. Studies show that $1,25(OH)_2D_3$ may activate protein kinase C (Merke *et al*, 1989; Yada *et al*, 1989; van Leeuwen *et al*, 1992; Khare *et al*, 1993, 1994; Bissonnette *et al*, 1995; Slater *et al*, 1995), modulate phospholipid metabolism (Morelli *et al*, 1993; de Boland *et al*, 1994), stimulate formation of cyclic nucleotides (Barsony and Marx, 1988, 1991; Bellido *et al*, 1993; Khare *et al*, 1993, 1994), and trigger calcium transport (de Boland and Norman, 1990a, 1990b; Jones and Sharpe, 1994; Sergeev and Rhoten, 1995). To describe these phenomena, Norman and Nemere proposed the term "nongenomic response" to emphasize the independence of the classic transcription-dependent signaling of steroid hormones (Nemere and Norman, 1987; Norman *et al*, 1992). The nongenomic mechanism has been claimed to be responsible for transcaltachia (rapid intestinal calcium transport) in mammals (Nemere and Norman, 1987; de Boland and Norman, 1990a), but it has not been clear whether the nongenomic events play any role in the regulation of cell proliferation and differentiation.

Involvement of nongenomic mechanisms has been postulated in the regulation of growth of keratinocytes by $1,25(OH)_2D_3$. Yada *et al* (1989) and our own results (Gniadecki, 1994) documented a rapid stimulation of protein kinase C activity in epidermal cells by $1,25(OH)_2D_3$ and its analog KH 1060. Jones and Sharpe (1994) demonstrated a rapid increase in intracellular calcium concentration after exposure to $1,25(OH)_2D_3$ and a variety of vitamin D analogs, but other investigators were unable to find evidence for either protein kinase C involvement (Koizumi *et al*, 1991) or stimulation of calcium currents (Pillai *et al*, 1995).

We have recently observed that the direction of the proliferative response to $1,25(OH)_2D_3$ in cultured human keratinocytes depends on the extracellular calcium concentration and the degree of cell differentiation (Gniadecki, 1996). In cells actively proliferating in media containing low calcium concentrations, treatment with $1,25(OH)_2D_3$ imposes a proliferative block in G_1 phase of the cycle. In contrast, in cells induced to differentiate by elevation of calcium concentration, $1,25(OH)_2D_3$ stimulates rather than inhibits DNA synthesis. Using this model, we investigated whether any rapid nongenomic events are induced by $1,25(OH)_2D_3$ in keratinocytes.

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Reprint requests to: Dr. Robert Gniadecki, Department of Dermatological Research, Leo Pharmaceutical Products, Industriparken 55, 2750 Ballerup, Denmark.

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; MAPK, mitogen-activated protein kinase; VDR, vitamin D receptor.

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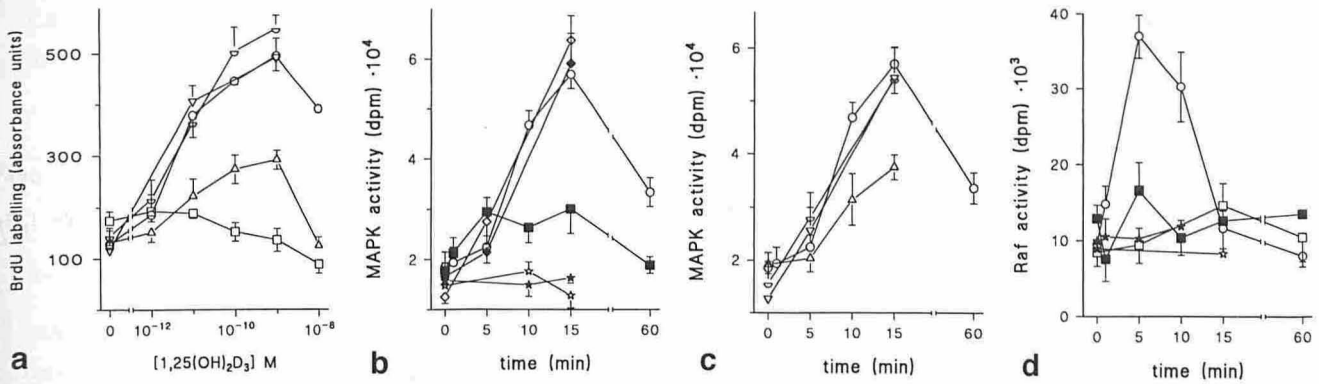

Figure 1. Stimulation of DNA synthesis and activation of MAPK and Raf by 1,25(OH)$_2$D$_3$. Normal human keratinocytes were preincubated in media containing 0.15 mM $CaCl_2$ (*squares*), 1.8 mM $CaCl_2$ (*circles*), or 1.8 mM $CaCl_2$ with the following: actinomycin D (*diamonds*), tyrphostin (*stars*), antisense *raf* oligomers (*triangles*), nonsense oligonucleotides (*lower semicircles*), or sense *raf* oligonucleotides (*inverted triangles*). The calcium concentration was adjusted 27–30 h before addition of 1,25(OH)$_2$D$_3$; 0.1 μM tyrphostin or 5 μg/ml actinomycin D was added 2 h (*open symbols*) or 24 h (*closed symbols*) before the addition of 1,25(OH)$_2$D$_3$. Cells were preincubated with antisense or sense *raf* oligomers 24 h before the addition of 1,25(OH)$_2$D$_3$. *a*, synthesis of DNA (measured as incorporation of BrdU) in keratinocytes 24 h after exposure to different concentrations of 1,25(OH)$_2$D$_3$ and tyrphostin or actinomycin D or *raf* oligomers. *b–d*, lysates from the cells treated with 1,25(OH)$_2$D$_3$ (10^{-9} M) for indicated times were immunoprecipitated with anti-MAPK (*b* and *c*) or anti-Raf (*d*) antibodies, and kinase activity of the immunoprecipitate was measured using myelin basic protein as a substrate. The effects of tyrphostin and actinomycin D on MAPK are shown in *b*; *c* illustrates inhibition of MAPK activation by antisense *raf* oligomers. Mean values (n = 3) with SD are shown.

Because activation of Raf kinase mitogen-activated protein kinases (MAPKs) is essential for stimulation of growth in many types of cells (Pelicci *et al*, 1992; Blenis, 1993; Egan *et al*, 1993; Laird *et al*, 1995), we asked whether Raf and MAPK are activated by 1,25(OH)$_2$D$_3$.

MATERIALS AND METHODS

Keratinocyte Culture Cryopreserved normal human keratinocytes were purchased from Clonetics (San Diego, CA) and cultured in keratinocyte basal medium (Clonetics) containing 0.15 mM $CaCl_2$, 1 ng/ml recombinant human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 0.1% bovine pituitary extract. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged at 80–90% confluence after trypsinization with 0.2 ml/cm^2 0.05% trypsin with 0.02% ethylenediamine tetraacetic acid solution, and were seeded at a density of 2500 cells/cm^2. Second- and third-passage cells were used for the studies. For bromodeoxyuridine (BrdU) labeling, cells were transferred to the medium without epidermal growth factor and antibiotics for 48 h. The calcium concentration was adjusted 27–30 h before the addition of 1,25(OH)$_2$D$_3$. Tyrphostin (final concentration 0.1 μM; Life Technologies, Gaithersburg, MD) or actinomycin D (5 μg/ml; Sigma, St. Louis, MO) was added 2 h or 24 h before the addition of 1,25(OH)$_2$D$_3$. Antisense *raf* oligomers of the sequence described by Riedel *et al* (1993) were used to downregulate Raf protein expression, and nonsense and sense oligomers were used as controls. In the preliminary experiments, a maximal downregulation of Raf protein (by approximately 90%, as assessed by Western blotting) was obtained with 30 μM antisense *raf* oligonucleotides after 24 h of incubation with the cells. These conditions were used in the present study. BrdU labeling was performed with the Cell Proliferation Kit (Amersham International). Four hours before termination of an experiment, BrdU at a final dilution 1:500 was added to the wells. Cells were washed extensively with phosphate-buffered saline, fixed in a mixture of 90% ethanol with 5% acetic acid, and blocked with 5% bovine serum albumin (fraction V) in phosphate-buffered saline supplemented with 0.1% Tween 20. BrdU-positive nuclei were labeled with the anti-BrdU antibody and detected with the peroxidase-labeled second antibody by enzyme-linked immunosorbent assay, according to the procedure described by the producer. Control experiments showed negative enzyme-linked immunosorbent assay reactions in samples incubated with the second antibody only.

Immunoprecipitation For immunoprecipitation in denatured conditions, the cells were dissolved in boiling 10 mM Tris HCl, pH 7.4, with 1% sodium dodecyl sulfate. To analyze the protein-protein interactions, we used the native immunoprecipitation technique, whereby the cells were lysed for 1 h at 4°C in the lysis buffer (10 mM Tris HCl, pH 7.4, 1% Triton X100, 0.5% Nonidet P-40, 150 mM NaCl, 20 mM NaF, 0.2 mM Na_3VO_4, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 20 μg/ml pepstatin). Aliquots containing 0.4 mg (denatured) and 1 mg (native) of protein were precleared with 20 μl of 50% slurry of protein A-Sepharose in the lysis buffer for 30 min at 4°C and immunoprecipitated with 3 μg of the appropriate antibody for 24 h and the same amount of the secondary antibody for 1 h at 4°C. Complexes were adsorbed to protein A-Sepharose given in the above quantity, washed three times with the lysis buffer, and analyzed with western blotting (Laemmli, 1970) using enhanced chemiluminescent detection (Amersham International, Little Chalfont, U.K.). To minimize the possibility of nonspecific immunoprecipitation, we reversed the sequence of addition of antibodies in some experiments, and assessed the purity of the immunoprecipitates in a Coomassie blue–stained gel. In the latter instance, only two protein bands originating from denatured immunoglobulin heavy and light chains were usually seen. The concentration of specifically precipitated proteins was too low for visualization with this method.

The following antibodies were used: anti-phosphotyrosine (clone PY20), anti-Shc, anti-Grb2, anti-mSos (all murine monoclonal antibodies; Transduction Laboratories, Lexington, KY); and anti-VDR rat monoclonal (clone 9A7γ; Chemicon, Temecula, CA). Secondary antibodies, rabbit anti-mouse, and rabbit anti-rat were obtained from Dako (Glostrup, Denmark). For detection in western blotting, appropriate horseradish peroxidase–labeled second antibodies were used (Dako).

Kinase Assays Cells were lysed in the lysis buffer for 60 min at 4°C. Aliquots of supernatant containing 100 μg of protein were immunoprecipitated in native conditions with 3 μg of monoclonal anti-MAPK antibody recognizing both ERK1 and ERK2 (Zymed, San Francisco, CA) or polyclonal goat anti-Raf antibody (Affiniti, Nottingham, UK) for 1 h, and for an additional 30 min with 3 μg of rabbit anti-mouse and rabbit anti-goat immunoglobulins, respectively (both from Dako). The immune complexes were collected on protein A-Sepharose (Pharmacia, Uppsala, Sweden), washed three times in the lysis buffer and three times with the kinase buffer (10 mM Tris HCl, pH 7.2, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 20 μg/ml pepstatin), and incubated at 37°C for 15 min with 40 μl of the kinase buffer containing 25 μM adenosine triphosphate (ATP), 2.5 μCi of [^{32}Pγ]ATP (Amersham International), and 1 mg/ml myelin basic protein, which is a substrate for both MAPK and Raf (Force *et al*, 1994). Phosphorylated product separated from the free isotope on phosphocellulose filters (Pierce, Rockford, IL) was counted in a scintillation counter.

RESULTS

Activation of MAPK and Raf by 1,25(OH)$_2$D$_3$ In keratinocytes grown in 0.15 mM calcium, 1,25(OH)$_2$D$_3$ imposed a proliferative block, but in cells pretreated in media containing 1.8 mM

calcium, growth stimulation was observed after exposure to $1,25(OH)_2D_3$ in low concentrations (10^{-11} to 10^{-9} M) **(Fig 1*a*)**. To investigate whether MAPK is activated by $1,25(OH)_2D_3$, we immunoprecipitated MAPK from cell lysates and assayed immunocomplexes for kinase activity in the presence of myelin basic protein as a substrate **(Fig 1*b,c*)**. In confluent cultures preincubated with 1.8 mM $CaCl_2$, but not in those cultured with 0.15 mM $CaCl_2$, $1,25(OH)_2D_3$ rapidly stimulated MAPK activity.

MAPK is phosphorylated by MAPK kinase MEK, which in turn is activated by Raf kinase (Egan *et al*, 1993). To see whether $1,25(OH)_2D_3$ stimulates Raf kinase, we performed a similar immunocomplex kinase assay using polyclonal anti-Raf antibodies. As shown in **Fig 1*d***, Raf kinase activity was stimulated after approximately 5 min of exposure to $1,25(OH)_2D_3$. Activation of MAPK and Raf preceded the increase of DNA synthesis, suggesting a causal relationship between these processes. To address this possibility, expression of Raf protein was inhibited by antisense oligonucleotides **(Fig 1*a,c*)**. Selective knockout of Raf reduced both the induction of MAPK activity and DNA synthesis, indicating that the Raf-MAPK pathway was directly involved in the stimulation of keratinocyte proliferation by $1,25(OH)_2D_3$. To investigate whether vitamin D stimulates MAPK through a transcription-dependent mechanism, we incubated keratinocytes with $1,25(OH)_2D_3$ in the presence of RNA and protein synthesis inhibitors (actinomycin D, **Fig 1**; and cycloheximide, not shown) and tested cell lysates for MAPK activity. These inhibitors did not prevent the activation of MAPK. Thus, activation of MAPK by $1,25(OH)_2D_3$ did not involve the known VDR-mediated mechanisms that are dependent on transcription and protein synthesis.

$1,25(OH)_2D_3$ Induces Phosphorylation of Shc and Interaction Between Shc and Grb2 Activation of the Raf-MAPK axis takes place when a receptor protein becomes phosphorylated on a tyrosine residue, or when an occupied receptor interacts with a nonreceptor tyrosine kinase that phosphorylates Shc proteins (Pelicci *et al*, 1992; Egan *et al*, 1993; Taniguchi, 1995). Phosphotyrosine-containing proteins bind to the proteins Grb2 and mSos, the latter of which stimulates guanosine triphosphate loading onto Ras and further activation of Raf. We therefore asked whether a similar sequence of events occurs in keratinocytes after application of the vitamin D hormone. Activation of MAPK was clearly dependent on the tyrosine kinase activity, as tyrosine kinase inhibitors (tyrphostin, **Fig 1**; and genistein, not shown) blocked MAPK activation in response to $1,25(OH)_2D_3$.

To evaluate the role of Shc in vitamin D signaling, we determined whether Shc becomes tyrosine phosphorylated after exposure of the cells to $1,25(OH)_2D_3$ **(Fig 2)**. Phosphotyrosine-containing proteins were immunoprecipitated with a monoclonal antibody, separated by polyacrylamide gel electrophoresis, and immunoblotted with anti-Shc antibody. To exclude nonspecific results, control experiments were done with the reverse order of added antibodies: Cell lysates were precipitated with anti-Shc antibody and blotted with anti-phosphotyrosine antibody. The results showed that $1,25(OH)_2D_3$ provoked tyrosine phosphorylation of $p66^{Shc}$, and to a lower extent $p52^{Shc}$. Phosphorylation of $p66^{Shc}$ was detected in both calcium-pretreated and nonpretreated cells.

Next we studied whether Shc forms complexes with Grb2 and mSos. Lysates from cells treated with 10^{-9} M $1,25(OH)_2D_3$ were immunoprecipitated in native conditions with anti-Shc antiserum and immunoblotted with anti-Grb2 antibody, and *vice versa* **(Fig 3*a,b*)**. Grb2 was found to associate with Shc selectively in the lysates from calcium-pretreated cells. Further, mSos was present in these complexes, as $p66^{Shc}$ could be detected in mSos precipitates from lysates of keratinocytes preincubated with 1.8 mM $CaCl_2$ and stimulated for 3 or 5 min with $1,25(OH)_2D_3$ **(Fig 3*c*)**. Surprisingly, an association between mSos and Grb2 was also induced by $1,25(OH)_2D_3$ in cells pretreated in high-calcium media **(Fig 3*d*)**.

Association of VDR and Shc Upon Treatment With $1,25(OH)_2D_3$ To determine whether VDR associates with Shc, we immunoprecipitated cell lysates with anti-Shc and anti-VDR antibodies and probed with anti-VDR and anti-Shc antibodies, respectively **(Fig 4)**. These experiments showed that VDR protein co-precipitates with Shc in cells stimulated with $1,25(OH)_2D_3$.

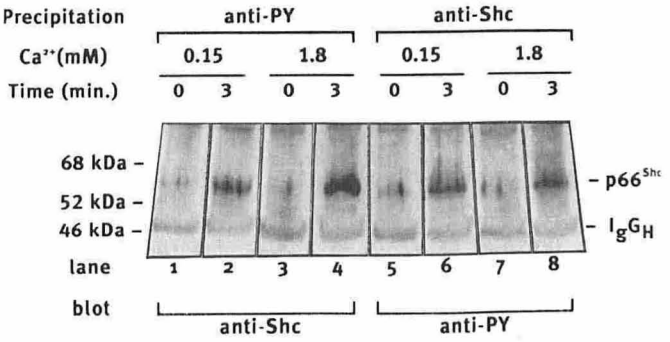

Figure 2. Tyrosine phosphorylation of Shc in response to $1,25(OH)_2D_3$. Keratinocytes were preincubated for 27–30 h in media with 0.15 mM or 1.8 mM $CaCl_2$ and exposed to 10^{-9} M $1,25(OH)_2D_3$ for indicated times. Cell lysates were immunoprecipitated in denatured conditions with anti-phosphotyrosine antibodies (*lanes 1–4*) or anti-Shc antibodies (*lanes 5–8*) and probed with anti-Shc or anti-phosphotyrosine antibodies, respectively. Positions of $p66^{Shc}$ and immunoglobulin heavy chain (IgG_H) are indicated. A fainter line below $p66^{Shc}$ represents $p52^{Shc}$; $p46^{Shc}$ could not be visualized because of a positional overlap with IgG_H. The experiment was repeated three times with identical results.

DISCUSSION

The data presented here suggest that $1,25(OH)_2D_3$ triggers a signaling cascade, involving sequentially (i) the interaction of VDR with Shc; (ii) tyrosine phosphorylation of Shc and complex formation between Shc, Grb2, and mSos; (iii) activation of Raf kinase; and (iv) activation of MAPK. This novel signaling pathway is illustrated schematically in **Fig 5**. The effects of $1,25(OH)_2D_3$ may be classified as nongenomic, as mRNA and protein synthesis inhibitors did not abrogate the activation of MAPK. In addition, the stimulation of Raf and MAPK was rapid, which virtually rules out the possibility that $1,25(OH)_2D_3$ induces the synthesis of an autocrine growth factor which then activates MAPK by binding to and activating cell-surface receptors.

The activation of MAPK was clearly dependent on and preceded by an activation of Raf. In accordance with the present results, Lissoos *et al* (1993) reported that $1,25(OH)_2D_3$ stimulates Raf activity in hepatic Ito cells and proposed that this mechanism is responsible for induction of proliferation in this cell type. Our experiments with antisense *raf* oligomers confirm the conclusions of Lissoos *et al* by showing that a knockout of Raf protein diminishes MAPK activation and reduces considerably the proliferative response of keratinocytes to $1,25(OH)_2D_3$. We were unable, however, to block the proliferative response and MAPK activation completely in this experiment. The existence of alternative pathway(s) stimulating MAPK, such as the recently described MAPKK cascade (Chao *et al*, 1994; Yan *et al*, 1994) or a residual Raf activity, could account for this fact.

The MAPK pathway is usually initiated by the phosphorylation of Shc proteins and their association with Grb2 and mSos, leading to the activation of Ras. The same sequence of events was demonstrated here in keratinocytes exposed to $1,25(OH)_2D_3$, suggesting a functional relationship between Shc phosphorylation and the formation of a protein complex composed of Shc, Grb2, and mSos. Unexpectedly, $p66^{Shc}$ seems to be mainly involved, which contrasts with the preferential involvement of $p46^{Shc}$ and $p52^{Shc}$ during activation of growth factor or cytokine receptors.

In many kinds of cells, the Grb2-mSos complexes preexist, and only the association between Shc and Grb2 is triggered by extracellular stimuli. We were unable to detect native Grb2-mSos complexes in keratinocytes, but their formation was initiated by treatment with $1,25(OH)_2D_3$. This result is not necessarily in conflict with the preexistence of Grb2-mSos complexes in kerati-

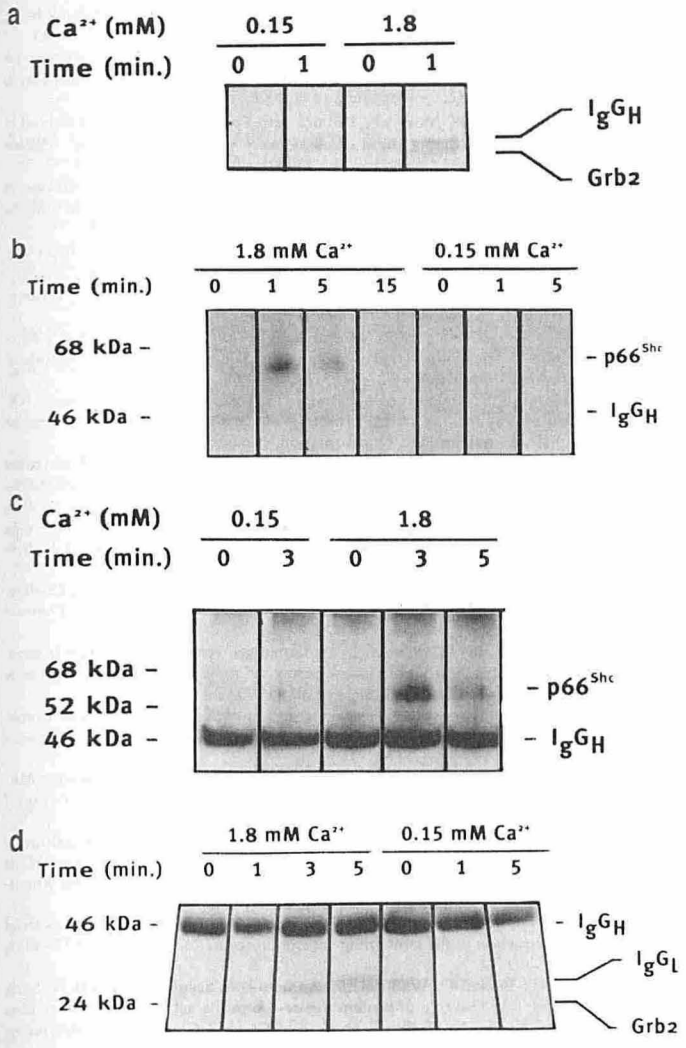

Figure 3. Formation of complexes between Shc, Grb2, and mSos in response to $1,25(OH)_2D_3$. Keratinocytes were preincubated for 27–30 h in media with 0.15 mM or 1.8 mM $CaCl_2$ and stimulated with 10^{-9} M $1,25(OH)_2D_3$ for indicated times. Cells were lysed and immunoprecipitated in native conditions with anti-Shc (*a*), anti-Grb2 (*b*), and anti-Sos (*c* and *d*) antibodies; western blotted; and probed with anti-Grb2 (*a* and *d*) and anti-Shc (*b* and *c*) antibodies. In *c*, a band between $p66^{Shc}$ and immunoglobulin heavy chain (IgG_H) represents $p52^{Shc}$. The experiment was repeated twice with identical results.

nocytes, but may simply indicate that complexes of Grb2 and mSos are too weak to remain intact during immunoprecipitation. A larger complex of Shc, Grb2, and mSos could be more stable, enabling its precipitation in the native conditions.

After exposure to $1,25(OH)_2D_3$, a complex formed between VDR and Shc. This was an unexpected finding because binding to Shc involves Src homology 2 domains (SH2) or phosphotyrosine-binding domains (Pelicci *et al*, 1992; Kavanaugh *et al*, 1995). VDR lacks SH2 and phosphotyrosine-binding domains, and it is likely that another protein is involved in bridging of VDR to Shc **(Fig 5)**. We are now in the process of identifying other protein components of the VDR-Shc complex.

A fully functional signaling cascade leading to the activation of MAPK and stimulation of DNA synthesis was triggered by $1,25(OH)_2D_3$ only in the keratinocytes preincubated in media containing an elevated calcium concentration. In the cells cultured in a low-calcium environment, $1,25(OH)_2D_3$ did not stimulate cell growth and MAPK was not significantly activated; however, the initial steps of the putative MAPK cascade, i.e., formation of the

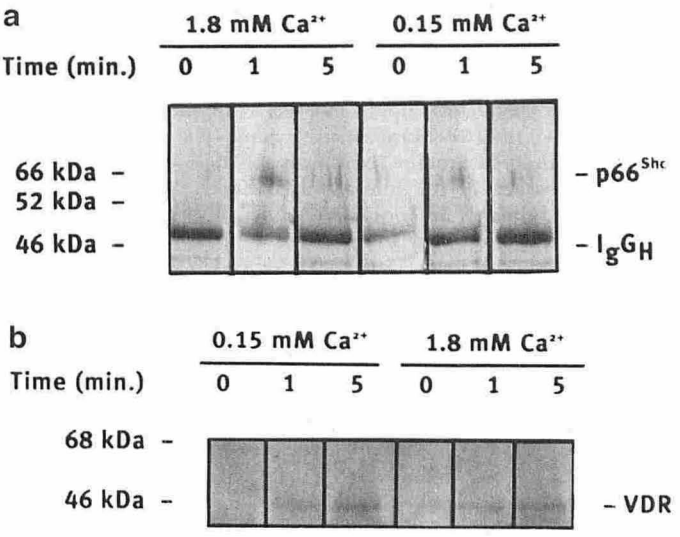

Figure 4. Formation of a complex between Shc and VDR in response to $1,25(OH)_2D_3$. Keratinocytes were preincubated for 27–30 h in media with 0.15 mM or 1.8 mM $CaCl_2$ and stimulated with 10^{-9} M $1,25(OH)_2D_3$ for indicated times. Cells were lysed and immunoprecipitated in native conditions with anti-VDR antibody (*a*) or anti-Shc antibody (*b*), western blotted, and probed with anti-Shc (*a*) and anti-VDR (*b*). In *a*, a band between $p66^{Shc}$ and immunoglobulin heavy chain (IgG_H) represents $p52^{Shc}$. The experiment was repeated three times with identical results.

VDR-Shc complex and Shc phosphorylation, took place in the latter instance. The cascade was interrupted downstream of the Shc phosphorylation step, precluding formation of Shc-Grb2 complexes in cells cultured in low-calcium media **(Fig 5)**. The nature of this inhibition remains unknown, but it is conceivable that an additional regulatory protein binding to Shc or Grb2 is involved. Alterna-

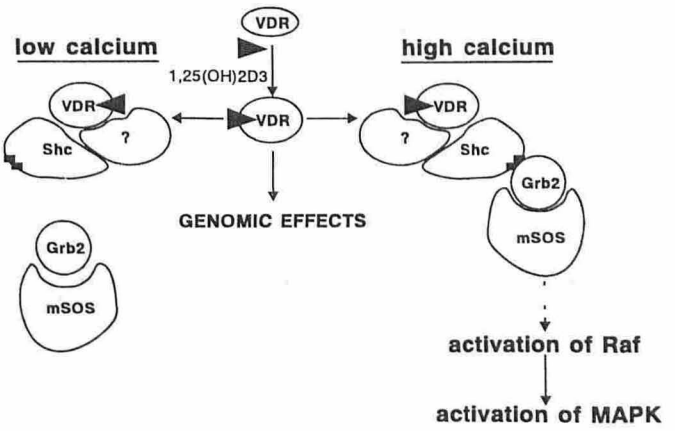

Figure 5. Proposed nongenomic signaling pathway for $1,25(OH)_2D_3$ in keratinocytes. After binding of $1,25(OH)_2D_3$ (*black triangle*), VDR associates with Shc, causing tyrosine phosphorylation (*black squares*) of the latter protein. It is likely that an additional unidentified protein mediates binding of VDR to Shc (*question mark*). In the cells exposed previously to media containing high calcium, phosphorylated Shc interacts with the proteins Grb2 and mSos, causing activation of Raf kinase, probably via activation of Ras. Raf kinase activates MAPK and eventually cell proliferation. In cells cultured with low concentrations of calcium, phosphorylated Shc does not interact with Grb2 and mSos, and thus the signaling cascade is interrupted and activation of MAPK does not take place. The role of the putative VDR-Shc-Grb2-mSOS complex in the activation of Raf has not been shown directly (*dashed line*).

tively, a protein inhibiting binding of phosphorylated Shc to Grb2 may be present in the cells grown in low-calcium conditions.

Taken together, our data suggest that $1,25(OH)_2D_3$ regulates the growth of keratinocytes by activating MAPK. Nevertheless, important pieces of evidence supporting the existence of a VDR-Shc-Grb2/mSos-Raf-MAPK cascade are lacking. The functional significance of observed protein complexes remains to be shown. The molecular nature of the interaction between VDR and Shc is obscure, and there is no conclusive evidence that VDR is directly responsible for phosphorylation of Shc. Stimulation of MAPK has not been observed for $1,25(OH)_2D_3$ or other steroid hormones, although events dependent on tyrosine phosphorylation have been described for progesterone (Mendoza *et al*, 1995). It appears that the MAPK pathway involves VDR, previously believed to be a transcription factor only. VDR may thus play a role in the nongenomic effects of $1,25(OH)_2D_3$, resembling the behavior of membrane growth factor receptors. In this context, it is interesting to note that VDR is phosphorylated *in vivo* (Darwish *et al*, 1993; Jurutka *et al*, 1993) and binds to the cytoskeleton upon exposure to the ligand (Barsony *et al*, 1990; Barsony and Marx, 1990), events that are also common to peptide hormone/growth factor receptors (Gronowski and Bertics, 1995). It is tempting to speculate that the cytoskeleton provides a site for interaction between $1,25(OH)_2D_3$ and MAPK, as both proteins have been found to bind to microtubules (Barsony *et al*, 1990; Reszka *et al*, 1995). The genomic responses elicited by steroid hormone receptors are modulated by and partially dependent on MAPK. Kato *et al* (1995) showed recently that the estrogen receptor is activated by phosphorylation of MAPK on a serine residue. Because VDR is also phosphorylated on a serine moiety *in vivo* (Jurutka *et al*, 1993), there is an intriguing possibility that the described MAPK activation may constitute an amplification mechanism for the genomic action of the vitamin D hormone.

Research shows that $1,25(OH)_2D_3$ activates multiple signaling pathways linked to protein kinase C, G proteins, or calcium channels. The MAPK cascade described here is therefore likely to represent only one of many pathways by which this hormone regulates cell growth. Recent evidence for the role of MAPK cascades in integrating distinct upstream signals (Schlaepfer *et al*, 1994; Maeda *et al*, 1995) may help to elucidate the complex and heterogeneous nongenomic events triggered in the cells by $1,25(OH)_2D_3$.

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